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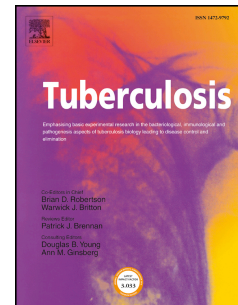
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Short communication (2500 words 15 refs 3 figures)

Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis

Oona Y-C. Lee^a, Houdini H.T. Wu^a, Gurdyal S. Besra^a, Bruce M. Rothschild^b, Mark Spigelman^c, Israel HersHKovitz^d, Gila Kahila Bar-Gal^e, Helen D. Donoghue^f and David E. Minnikin^{a,*}

^a Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK

^b Biodiversity Institute and Departments of Anthropology and Geology, University of Kansas, Lawrence KS 66045, USA

^c Kuvim Center for the Study of Infectious and Tropical Diseases and Ancient DNA, Hadassah Medical School, Hebrew University, Jerusalem, Israel

^d Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

^e Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel

^f Centres for Clinical Microbiology and the History of Medicine, University College London, UK

Email addresses

leeoy@bham.ac.uk; h.wu.2@bham.ac.uk; d.e.minnikin@bham.ac.uk; g.besra@bham.ac.uk; bmr@ku.edu; spigelman@btinternet.com; anatom2@post.tau.ac.il; gila.kahila@mail.huji.ac.il; h.donoghue@ucl.ac.uk

***Corresponding author**

Professor David E. Minnikin, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Email: d.e.minnikin@bham.ac.uk Telephone: +44(0)1214158126

SUMMARY

Studies on the evolution of tuberculosis, and the influence of this disease on human and animal development and interaction, require the accumulation of indisputable biomarker evidence. Ideally, the determination of full genomes would provide all the necessary information, but for very old specimens DNA preservation may be compromised and only limited DNA amplification may be a possibility. *Mycobacterium tuberculosis* is characterised by the presence of unusual cell envelope lipids, with specific biomarker potential. Lipid biomarker recognition has been decisive in pinpointing the oldest known cases of human and animal tuberculosis; the former are a woman and child from a pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka) and the latter is an extinct *Bison antiquus* from Natural Trap Cave, Wyoming (~17,000 ka). Including some new data, it is demonstrated how analysis of a combination of mycolic, mycocerosic and mycolipenic acid and phthiocerol biomarkers provide incontrovertible evidence for tuberculosis in these landmark specimens.

Keywords: Ancient tuberculosis; Lipids; Biomarkers

1. Introduction

Exploration of the origins and evolution of tuberculosis necessarily relies on the clear unambiguous identification of ancient well-characterised archaeological specimens. It would be advantageous to identify an extended population of infected individuals but, given the likely scarcity of the oldest examples, investigation of single landmark cases may well be a productive option. If studies of isolated cases are well-conducted and published in established peer-reviewed journals, the results achieved must be properly respected. In this report, the incontrovertible evidence for tuberculosis in the oldest human¹ and animal² cases, ~~described to date,~~ will be reviewed and some new data included.

The widest possible combination of complementary methods should be used to diagnose ancient mycobacterial disease. For skeletal material, considerable expertise has been developed in recognising characteristic bone changes linked to tuberculosis infection.^{3,4} The precise diagnosis of tuberculosis disease requires recognition of decisive biomarkers⁵ for the causative agent *Mycobacterium tuberculosis*. ~~The amplification of key DNA sequences has been systematically developed into widely applied protocols, during the past two decades~~ During the past twenty years, DNA fragment analysis has been extensively utilised.⁵ Major advances in determining full genomic data have been recently provided by the application of so-called “Next Generation Sequencing”⁶ and the more direct “Metagenomic” approach.⁷

The predominant feature of the tubercle bacillus is the presence of high proportions of long-chain lipids, easily distinguishable from any mammalian lipids. In a pioneering study, the 70 to 90 carbon mycolic acids (MAs) (Figure 1A) were clearly identified in a mediaeval bone from Addingham, UK, complementing DNA amplification and skeletal indications.⁸ The biomarker range has been extended to include multi-methyl-branched mycocerosic and mycolipenic acids (Figure 1B).^{2,9} MAs were originally analysed by fluorescence High Performance Liquid Chromatography (HPLC) of slightly unstable methylantryl esters,⁸ so a special robust derivatisation protocol, involving pyrenebutyrates of pentafluorobenzyl (PFB) esters was systematically developed.^{1,2,9} The mycocerosate and mycolipenate PFB esters can be exquisitely detected by Selected Ion Monitoring (SIM) Negative Ion Chemical Ionisation (NI-CI) Gas Chromatography Mass Spectrometry.^{2,9} ~~The value of lipid biomarkers in providing decisive confirmation of tuberculosis in key archaeological specimens will be reviewed and emphasised. The cases under consideration are~~ Lipid biomarker detection has been particularly decisive in diagnosing tuberculosis in ribs from a woman and child from a

pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka)¹ and an extinct *Bison antiquus* metacarpal from Natural Trap Cave, Wyoming (~17,000 ka).² However, these clear diagnostic data are occasionally overlooked⁴, so in this communication the data for these two landmark cases are presented together and reinforced by some new lipid results.

2. Landmark studies

2.1 Nine thousand year old woman and child, Atlit-Yam, Israel

Archaeological investigations off the coast at Atlit-Yam revealed a submerged coastal pre-pottery, post-domestication Neolithic settlement, which included skeletal material from a woman and infant with lesions suggestive of tuberculosis.^{1,10} In particular, the inner aspect of the infant cranial bones had serpentine engravings (*serpens endocrania symmetrica*, SES), considered to be diagnostic for intra-thoracic inflammation associated with tuberculosis.^{1,11} The tubular bones from the infant, and to a lesser extent from the adult, also demonstrated lesions identified as hypertrophic osteoarthropathy (HOA), highly suggestive of tuberculosis.^{1,12} Encouraging results were also achieved for the PCR amplification and sequencing of the *M. tuberculosis* DNA insertion elements IS6110 and IS1081,¹ but additional confirmation was desirable.

Carefully crafted robust lipid biomarker studies were found to be ideal complements to the above compelling evidence for TB in the Atlit-Yam skeletons.¹ The chosen bone samples were degraded by a proven alkaline hydrolysis,^{1,2,8} designed to release the maximum amount of mycobacterial lipid biomarkers. Quantitative conversion of the acidic fatty components to pentafluorobenzyl (PFB) esters gave lipid biomarkers the ability to be separated reproducibly on silica gel cartridges into fractions containing PFB mycolipenate/mycocerosates, PFB mycolates and free phthiocerols (Figure 1). The latter two lipid classes were converted to stable pyrenebutyric acid (PBA) esters, ideal for sensitive fluorescence HPLC, with the PFB mycocerosate/mycolipenates being amenable to NI-CI GC-MS. The assembled lipid biomarker profiles for the Atlit-Yam specimens are shown in Figure 2.

A simple logical sequence is followed for the HPLC characterisation of mycolic acid derivatives. The initial “reverse phase” HPLC (Figure 2A) serves to isolate and observe any C₇₀ – C₉₀ mycolates free from any smaller mammalian lipids. A “tight envelope” of total mycolate peaks, as recorded in Figure 2A, is very characteristic for members of the *M. tuberculosis* complex and, indeed, such clear profiles are immediately very positive indications of tuberculosis infections.^{5,8} The next stage is to subject the collected total

mycolates (Figure 2A) to “normal phase” HPLC to separate the α -, methoxy- and ketomycolate classes (Figure 1A) according to their polarity (Figure 2B); if clear peaks are seen for the individual mycolates, this strengthens the diagnosis of TB. Reverse phase HPLC of each of the collected mycolate types (Figure 2B) can provide very diagnostic profiles (Figure 2C-E). The α -mycolates are relatively homogeneous, a regular series of C₇₆ to C₈₄ homologues (Figure 2C) having two *cis*-cyclopropane rings (Figure 1A). The oxygenated mycolates, in contrast, comprise two overlapping homologous series with either *cis*- or *trans*-cyclopropane rings (Figure 1A). A very diagnostic feature of the methoxymycolates from *M. tuberculosis* (Figure 2D) is the presence of a double peak comprising the C₈₇ *cis*-cyclopropyl and C₈₈ *trans*-cyclopropyl methoxymycolates. Similarly, the ketomycolates from *M. tuberculosis* are characteristically dominated by the presence of the C₈₇ *trans*-cyclopropyl ketomycolate (Figure 2E). This sequential analysis enables a close correlation to be observed between the mycolate patterns for the Atlit-Yam specimens and standard *M. tuberculosis*.

To support the positive mycolate results, the mycocerosate/mycolipenate (Figure 1B) and phthiocerol family (Figure 1C) profiles have been prepared, in unpublished studies, using NI-CI GC-MS of PFB esters for the former (Figure 2F-I) and fluorescence HPLC of PBA esters for the latter (Figure 2J). Again, the mycocerosate/mycolipenate traces (Figure 2F-I) correspond well with those for standard *M. tuberculosis*. Interestingly, the infant showed a stronger presence of mycolipenate (Figure 2H, C₂₇ *m/z* 407). The presence of members of the phthiocerol family (Figure 1C) was revealed by reverse phase HPLC (Figure 2J) for ribs from the Atlit-Yam Infant and Woman. The main components corresponded to the characteristic *M. tuberculosis* C₃₄ and C₃₆ phthiocerol As (PA) and C₃₃ and C₃₅ phthiodiolones (PO); there was insufficient material to carry out confirmatory normal phase HPLC.

2.2. Seventeen thousand year old bison Natural Trap Cave Wyoming USA

Excavations directed by the late Larry D. Martin (1943-2013) of the Biodiversity Institute, University of Kansas, in Natural Trap Cave, Wyoming, revealed the presence of a variety of well-preserved Pleistocene animal bones^{2,3}. In particular, a metacarpal from a 17 ka extinct *Bison antiquus* showed evidence of lesions “undermining the articular surface”, associated with tuberculosis both in humans and animals.^{2,3} In a landmark study, aDNA characteristic of *M. tuberculosis* rather than *M. bovis* was clearly identified.¹²

A search for lipid biomarkers provided weak but encouraging profiles of mycolic acids (Figure 3A-E). Remarkably, absolutely pristine profiles of mycolipenic (C₂₇ *m/z* 407) and

mycocerosic acids (Figure 1B) were recorded for specimens taken both at the lesion (Bison 1) and a remote site (Bison 2) (Figure 3F-H). The initial reverse phase HPLC profiles of the PBA derivatives of the phthiocerol family fraction (Figure 3I) were not as clean as those for the Atlit-Yam skeletons (Figure 2J). Normal phase analysis (Figure 3J) of the collected material from the reverse phase separation (Figure 3I) confirmed the presence of members of the phthiocerol family, as published previously.² In addition, to the phthiocerols, however, the full normal phase profiles of the PBA derivatives showed the presence of ill-defined more-polar components, labelled X, Y and Z in Figure 3J. The presence of these unknown components was not reported in the initial study,² as their identity could not be readily understood. One possible explanation is that components X – Z are derived from glycosyl phenolphthiocerols, the deacylated so-called “phenolic” glycolipids (PGLs). To investigate this hypothesis, the PGL from *M. bovis* BCG was processed through the hydrolysis and extraction protocols and it was found that the resulting PBA derivatives chromatographed in a similar fashion to the components X, Y and Z (data not shown). Unfortunately, none of the X – Z material remained from the bison samples, so it was not possible to make a precise direct comparison. The key conclusion, however, is that the material from Atlit-Yam does not show the presence of these potential biomarkers, possibly derived from PGLs (Figure 2J). Many modern isolates of *M. tuberculosis* do not produce PGLs, but they are found in *M. bovis*, *M. africanum*, certain *M. tuberculosis* Beijing strains and smooth morphology isolates labelled “*Mycobacterium canettii*”. A recent detailed study¹³ has confirmed that “*M. canettii*” strains are likely ancient progenitors of modern tuberculosis. The aDNA studies¹² ruled out *M. bovis*, but indicated a similarity with *M. africanum*. Further studies will be needed to determine the precise strain of tubercle bacillus infecting the Natural Trap Cave bison.

3. Conclusions

Sound evidence has been assembled, therefore, for the clear diagnosis of tuberculosis in ~9ka human skeletons and a ~17ka bison metacarpal; these examples provide solid reference points for tracing the evolution of tuberculosis back into antiquity. Surprisingly, the actual validity of the aDNA results in the properly peer-reviewed Atlit-Yam study² has been directly challenged,¹⁴ with no mention being made of the very conclusive MA lipid biomarker evidence. Equally, in an otherwise generally comprehensive review on ancient tuberculosis and leprosy,⁴ the critique was repeated but no reference whatsoever was made to a range of published lipid biomarker results,⁵ even though the Atlit-Yam¹ and Addingham⁸ studies were cited.⁴ The inclusion of new mycocerosate/mycolipenate and phthiocerol data for the Atlit-

Yam specimens (Figure 2F-J) and additional components in the phthiocerol area for the Natural Trap Cave bison (Figure 3J) adds further weight to the power of lipid biomarkers in the diagnosis of ancient tuberculosis.

Genomic studies suggest a rapid expansion of modern biotypes of the tubercle bacillus following an apparent evolutionary bottleneck at the end of the Pleistocene.¹³ In a detailed examination of the parallel evolution of genomes from 186 members of the *M. tuberculosis* complex and 4,995 human mitochondria, extrapolations were interpreted to suggest that TB and humans co-evolved “out of Africa”, commencing ~70 ka ago.¹⁵ However, parallel evolution is not necessarily linked co-evolution and absolutely no evidence was advanced to substantiate the presence of any human tuberculosis going back to 70 ka. Indeed, not a single citation was made to any previous publication on the diagnosis of ancient tuberculosis in archaeological material! The current lack of any identified cases of Pleistocene TB in *Homo sapiens* and the apparent abundance of the disease in Pleistocene megafauna has led to the suggestion that animals may well have been prime vectors to facilitate the emergence of a viable tuberculosis pathogen from an environmental ancestor.²

Dedication: This paper is dedicated to the memory of Larry Martin (1943 – 2013), formerly of the Biodiversity Institute, University of Kansas, whose informed selection of specimens from Natural Trap Cave, Wyoming, led directly to the first diagnosis of tuberculosis in Pleistocene megafauna.

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Figure legends

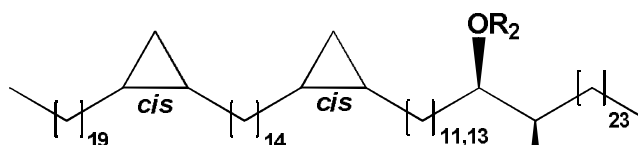
Figure 1. Lipid biomarkers for *M. tuberculosis*. (A) Generalized structures of α -, methoxy- and ketomycolates; the main components are in brackets. (B) Structures of mycolipenate and mycocerosates, showing negative carboxylate ions used for selected ion monitoring on NI-CI GC-MS analysis of pentafluorobenzyl (PFB) esters. (C) Structures of members of the phthiocerol family.

Figure 2. Mycobacterial lipid profiles from standard *M. tuberculosis* and Atlit-Yam Woman and Infant. (A – E) Reverse and normal phase fluorescence HPLC of mycolate pyrenebutyric acid (PBA) derivatives of pentafluorobenzyl (PFB) esters. (F –I) NI-CI GC-MS of pentafluorobenzyl (PFB) esters of mycolipenate and mycocerosates; relative intensities are shown normalised to the major component [100]. (J) Reverse phase fluorescence HPLC of pyrenebutyric acid (PBA) derivatives of members of the phthiocerol family: PA phthiocerol A, PB phthiocerol B, PO phthiodiolone.

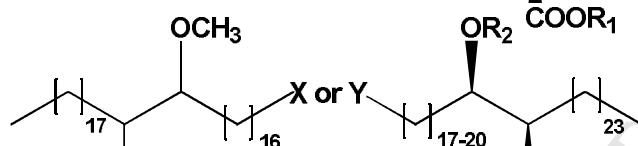
Figure 3. Mycobacterial lipid profiles from standard *M. tuberculosis* and Natural Trap Cave Bison metacarpal specimens. (A – E) Reverse and normal phase fluorescence HPLC of mycolate pyrenebutyric acid (PBA) derivatives of pentafluorobenzyl (PFB) esters. (F –H) NI-CI GC-MS of pentafluorobenzyl (PFB) esters of mycolipenate and mycocerosates; relative intensities are shown normalised to the major component [100]. (I and H) Reverse (I) and normal (J) phase fluorescence HPLC of pyrenebutyric acid (PBA) derivatives of components recovered with members of the phthiocerol family: PA phthiocerol A, PB phthiocerol B, PO phthiodiolone, X, Y & Z unknown components.

A. Mycolates

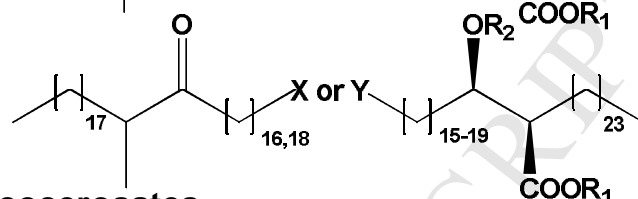
α -Mycolates
C76-82 (C78, 80)



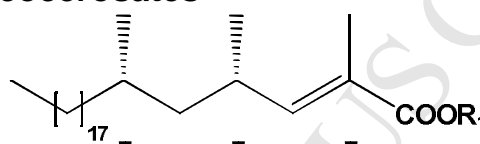
Methoxymycolates
C83-90 (C85)



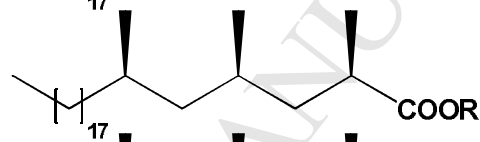
Ketomycolates
C84-89 (C87)

**B. Mycolipenate and mycocerosates**

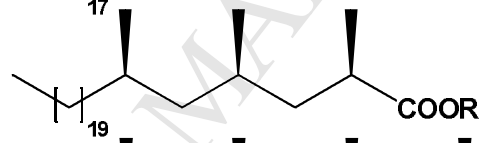
C27 Mycolipenate
m/z 407



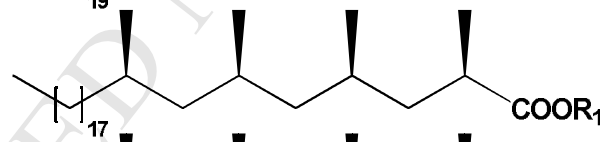
C27 Mycocerosate
m/z 409



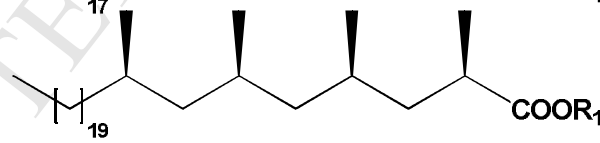
C29 Mycocerosate
m/z 437



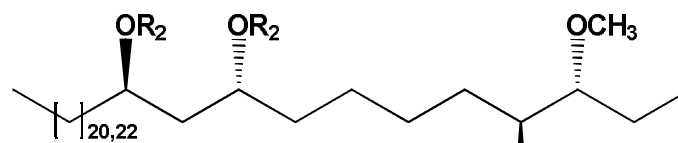
C30 Mycocerosate
m/z 451



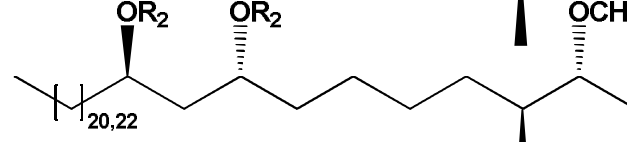
C32 Mycocerosate
m/z 479

**C. Phthiocerols**

Phthiocerol A
C34, 36



Phthiocerol B
C33, 35



Phthiodiolone
C33, 35

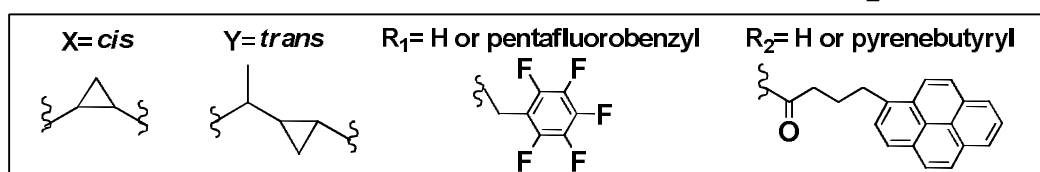
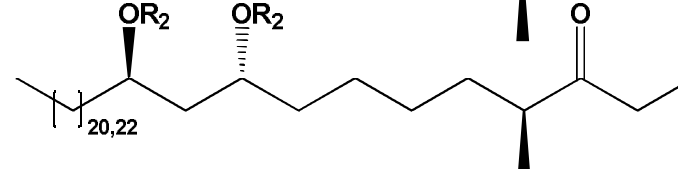


Figure 1

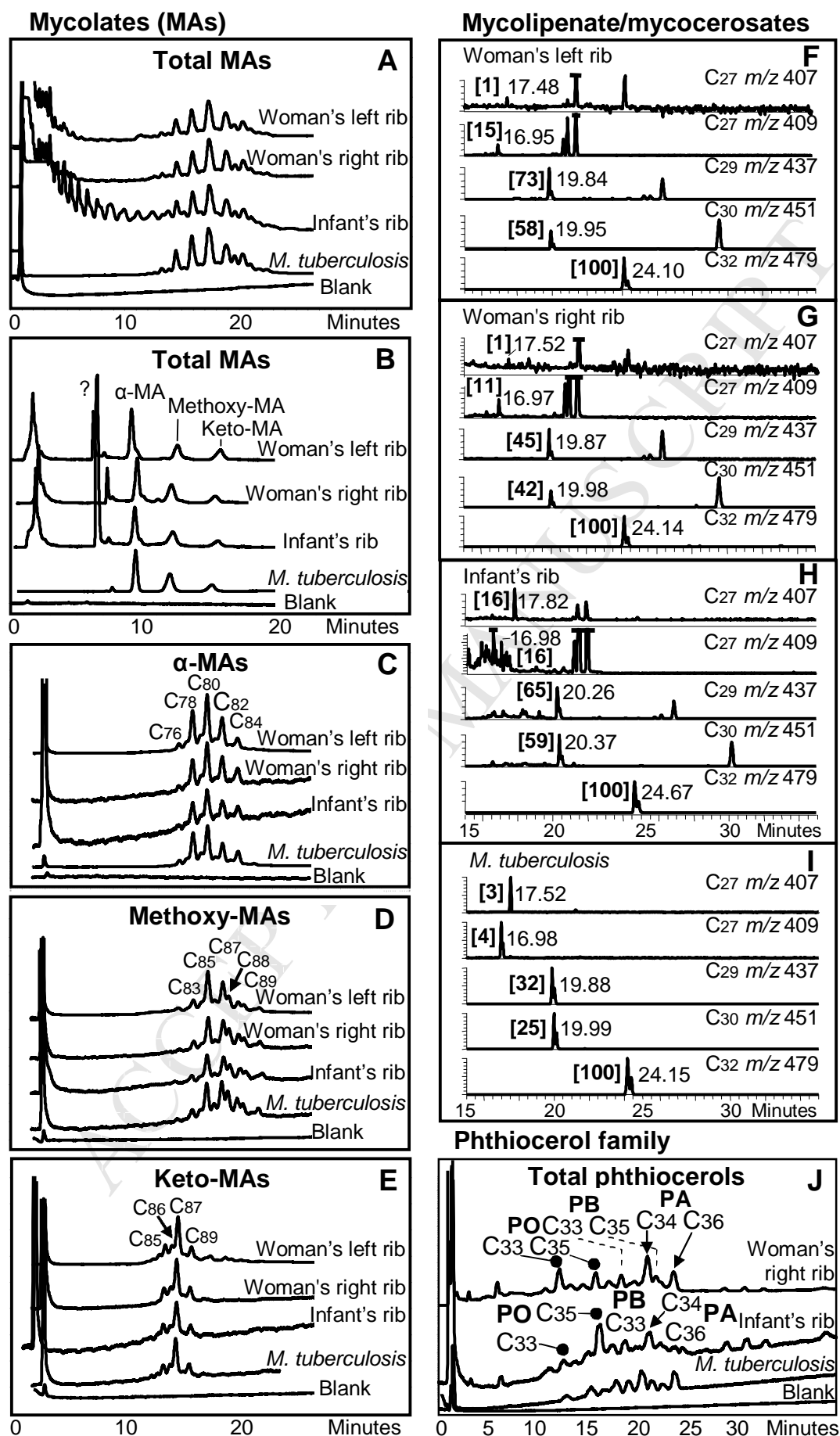


Figure 2

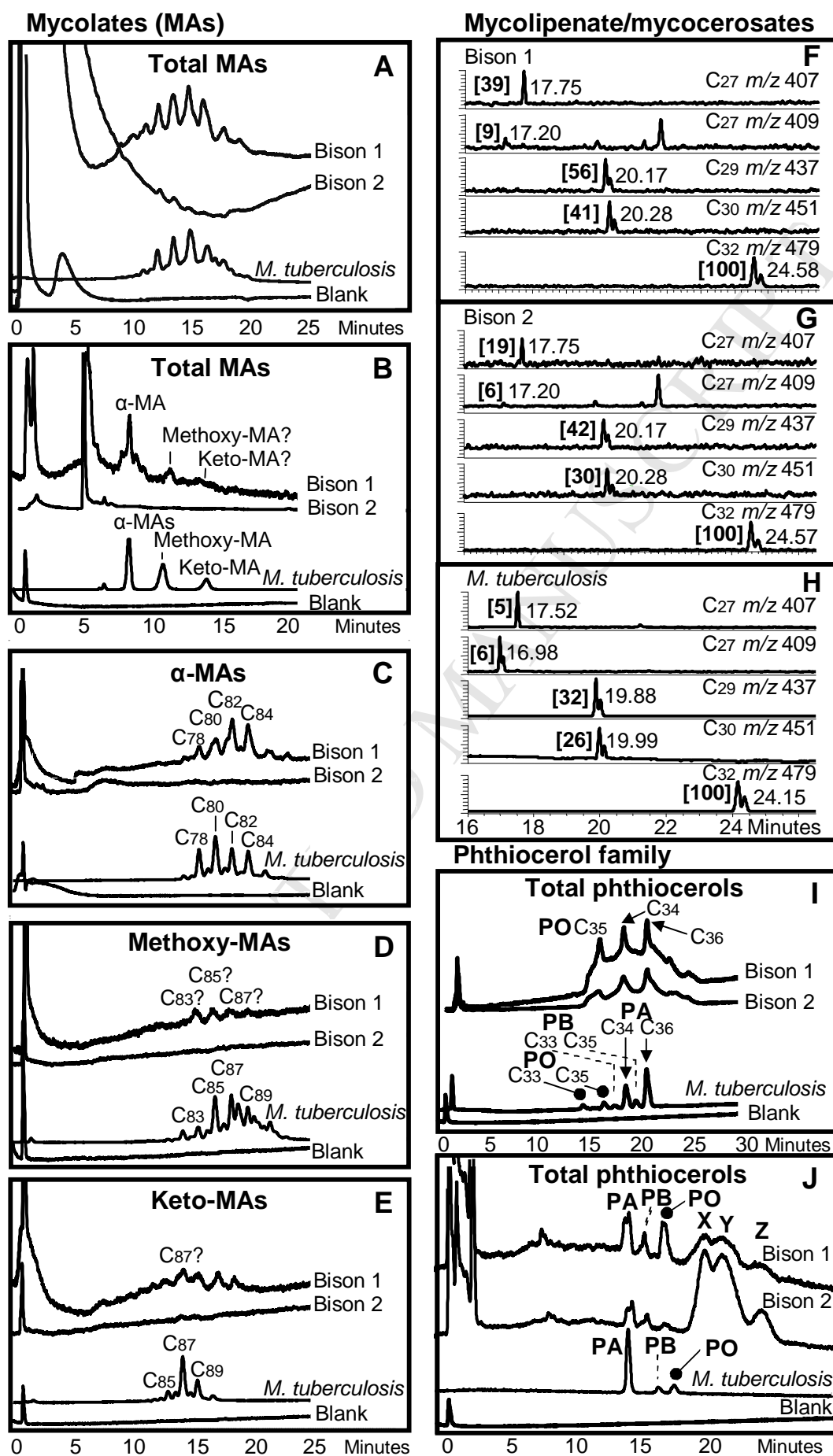


Figure 3